Molecular Studies on Celastrol-induced Bcr-Abl Oncoprotein Degradation in Human Leukemia Cells

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ABSTRACT

The chimeric Bcr-Abl oncprotein with constitutive tyrosine kinase activity plays a pivotal role in the pathogenesis of chronic myeloid leukemia (CML), therefore being an ideal target for the drug development. Although tyrosine kinase inhibitors achieved great success in the treatment of CML, their effect could be abrogated by mutation in the kinase domain where they bind to. Therefore inducing Bcr-Abl degradation could be an alternative strategy to treat Bcr-Abl-driven leukemia. Celastrol is a quinone methide triterpene with various biological activities including anticancer activity. In the current study, we reported that celastrol induced time-dependent Bcr-Abl degradation and apoptosis in K-562 CML cells. We also found that short-time exposure to celastrol was sufficient to induce committed apoptotic signal; removal of celastrol could cause re-expression of Bcr-Abl, which however cannot rescue the cell from undergoing apoptosis. In addition, we found that celastrol-induced Bcr-Abl degradation and cell apoptosis were not suppressed by commonly used protease inhibitors or their mixture under the selected experimental conditions. Our study clearly demonstrated that celastrol inhibits Bcr-Abl expression/function by inducing its degradation, which at least contributes to our understanding of this natural product’s ability to induce tumor cell apoptosis. Our finding strongly supports the idea that celastrol could be developed into a novel anti-CML drug.

INTRODUCTION

More than 90% of chronic myeloid leukemia (CML) is associated with a chromosomal translocation t(9;22)(q34;q11) known as the Philadelphia chromosome, which was also found in some acute leukemia [1]. During this process, part of the bcr gene from chromosome 22 is fused with the c-abl gene on chromosome 9, resulting in a chimeric oncogene, bcr-abl [1-2]. The fused Bcr-Abl protein has constitutively elevated tyrosine kinase activity and acts as a proliferative activator and an apoptotic suppressor [1-2]. Bcr-Abl is linked to Ras and PI3K pathways, focal adhesion complexes, and messenger systems such as Jak-Stat kinases [1-2]. In addition, Bcr-Abl can abrogate growth factor dependence, enhance DNA damage repair, and indirectly modulate Bcl-2 family proteins [1-2]. Inhibition of Bcr-Abl tyrosine kinase activity by kinase inhibitors such as imatinib
has achieved great success in the treatment of Bcr-Abl-driven leukemia in the past decade [3-4]. However, single point mutation in the Abl kinase domain may make Bcr-Abl escape from kinase inhibition [3-4]. Therefore another strategy to inhibit Bcr-Abl function is to reduce the amount of Bcr-Abl protein by either decreasing its synthesis or promoting its degradation. Knocking down Bcr-Abl by RNA interference effectively induced apoptosis and reduced viability in human K-562 cell line and primary CML cells [5-6]. We and others have also reported that treatment with proteasome inhibitors caused significant reduction of Bcr-Abl protein, associated with subsequent induction of apoptosis in K-562 cells [7].

Celastrol, a quinone methide triterpene, is isolated from the root bark of Thunder God Vine (*Tripterygium wilfordii*) Hook F., TWFH, a perennial vine of Celastraceae family (bittersweet) [8-9]. Celastrol has exhibited promising anticancer activity in different cancer cells including leukemia and solid tumors both in vitro and in vivo [8-9]. It can inhibit cancer cell proliferation, induce apoptosis, prevent their malignant tissue invasion and suppress tumor angiogenesis [8-9]. It has also been reported that celastrol is able to eradicate acute myeloid leukemia at the progenitor and stem cell level [10]. Several molecular targets of celastrol have been identified, including HSP90 [11], NF-κB [12] as well as proteasome [13]. Besides, celastrol has been reported as a chemosensitizer and a radiosensitizer. It can sensitize resistant melanoma cells to the effect of temozolomide, an alkylating agent, in a synergistic manner [14]. It also potentiates radiotherapy in hormone-refractory prostate cancer cells by impeding DNA damage repair and augmenting apoptosis [15]. Recently, we also reported that celastrol exhibited potent chemosensitizing activity in K-562 leukemia cells, associated with decreased level of Bcr-Abl oncoprotein [16].

In the current study, we investigated the molecular mechanism on celastrol-mediated inhibition of Bcr-Abl protein expression. Here, we reported that (i) celastrol induced apoptosis and Bcr-Abl degradation in a time-dependent manner; (ii) celastrol-induced apoptosis was not blocked by newly synthesized Bcr-Abl protein, once the cells were committed; (iii) celastrol-induced Bcr-Abl degradation and apoptosis were not prevented by selected protease inhibitors or their mixture under the selected experimental conditions. These findings shed light on the mechanism how celastrol inhibits Bcr-Abl protein expression/function and provide support for the potential use of celastrol in the CML treatment.

**MATERIALS AND METHODOLOGY**

**Materials**

Celastrol was purchased from Cayman Chemicals (Ann Arbor, MI). The antibody against c-Abl was from Cell Signaling (Beverly, MA); antibodies against PARP, tubulin and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Cycloheximide solution, CA074-Me, aprotinin, leupeptin, and N-ethylmaleimide (NEM) were from Sigma-Aldrich (St Louis, MO). Pepstatin was ordered from Roche (Mannheim, Germany). All these reagents were prepared according to the manufacturer’s instructions.

**Cell culture**

The human chronic myeloid leukemia K-562 cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Aleken Biologicals, Nash, TX), 100 Units/mL of penicillin, 100 μg/mL of streptomycin and 0.3 mg/mL L-glutamine (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO2.

**Cell number counting and whole cell extract preparation.**

Total cell numbers were counted manually by hemocytometer. Whole cell extract was prepared using lysis buffer (50 mM Tris-HCl/pH 8.0, 150 mM NaCl, 0.5% NP-40) as described previously. The protein concentrations were determined by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

**Caspase-3 activity assay.**

Fresh-made whole cell extract (20 μg per sample) was incubated with 20 μM fluorogenic caspase-3 substrate Ac-DEVD-AMC (Calbiochem, La Jolla, CA) in 100 μL of Tris-HCl (20 mM, pH 7.5). After 2 hours incubation at 37°C, the AMC liberated from the fluorogenic substrate was detected spectrofluorometrically (λex = 355 nm and λem = 460 nm).
Western blot analysis.

Whole cell extract (40 μg per sample) was separated by 6% SDS-PAGE, transferred to a nitrocellulose membrane, immunoblotted with indicated antibodies, and detected by HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ). Densitometry was quantified using AlphaEase FC software (Alpha Innotech, San Leandro, CA).

RNA extraction and RT-PCR.

Total RNA from cells was extracted using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). RNA concentration was determined spectrophotometrically. Total RNA (2 μg) was reverse transcribed with random hexamer primers using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Four μL of the cDNA product was used as template for PCR amplification with 0.2 μM gene specific primers using Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). The primers for Bcr-Abl were 5'-TTCAGAGCTTCTCCGTGACAT-3' and 5'-TGTTGACTGGCTGAGTTGCTTG-3'. The PCR condition for Bcr-Abl was 10 min at 96 °C, followed by 35 cycles of 1 min at 96 °C, 1 min at 64 °C and 1 min at 72 °C. The primers for GAPDH were 5'-TTGCAACTGTTTCTGAGC-3' and 5'-AGCATTGGAAAATGTTCAAAG-3'. The PCR condition for GAPDH was 10 min at 95°C, followed by 30 cycles of 1 min at 95 °C, 1 min at 54 °C and 1 min at 72 °C. Different PCR cycle numbers were tested for Bcr-Abl and GAPDH to ensure that the assay was in the linear range of amplification. The PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. Densitometry was quantified using AlphaEase FC software (Alpha Innotech, San Leandro, CA).

Protease inhibitor experiment.

K-562 cells were pretreated with either DMSO, CA074-Me (0.5 μM), pepstatin (15 μM), aprotinin (10 μM), leupeptin (400 μM) or NEM (15 μM), or a mixture of the above five inhibitors at the indicated concentrations for 4 hours, followed by treatment with 1 μM celastrol for 12 hours. Cells were then collected for caspase-3 activity assay and Western Blot analysis.

RESULTS AND OBSERVATIONS

Celastrol induced apoptosis and Bcr-Abl degradation in a time-dependent manner.

We previously reported down-regulation of Bcr-Abl protein expression by celastrol treatment [16]. In order to explore the involved molecular mechanism, we performed a kinetic experiment. K-562 cells were treated with 1 μM celastrol for up to 36 hours which is about 1.5-fold of the doubling time of these cells. A dramatic decrease of cell number was observed in the celastrol-treated group compared to the DMSO control group at the ending time point (Fig. 1A). Specifically, celastrol-treated cells decreased ~55% while control cells increased ~60% in cell number (Fig. 1A). Caspase-3 activation occurred at as early as 4 hours under the experimental condition, with a peak at 12 hours (Fig. 1B). The decrease of caspase-3 activity at 24 and 36 hours was most possibly due to the loss of cells or the formation of apoptotic bodies which might not be included in our cell lystate preparation. Consistently, poly(ADP-ribose) polymerase (PARP) cleavage occurred at 4 hours (Fig. 1C). A decrease in the amount of full-length PARP was associated with an increase in p85/PARP fragment (Fig. 1C). Most importantly, Bcr-Abl degradation also occurred at as early as 4 hours as manifested by the decrease of full length Bcr-Abl protein and the appearance of some new bands with lower molecular weight that might be its cleaved fragments (indicated by arrows; Fig. 1C). Unlike Bcr-Abl oncoprotein, normal Abl protein was only slightly decreased during celastrol treatment (Fig. 1C), suggesting that Bcr-Abl oncoprotein is much more sensitive to celastrol exposure than Abl kinase.

To test the possibility that suppression of Bcr-Abl transcription also contributes to its decrease at protein level, we measured the amount of Bcr-Abl mRNA in the same experiment by semi-qualitative RT-PCR analysis. Compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control, no significant change in the level of Bcr-Abl mRNA was detected at any time points (Fig. 1D). Taken together, these results indicate that celastrol induced Bcr-Abl protein degradation, associated with apoptosis induction in K-562 cells.
Induction of Bcr-Abl degradation by celastrol

Apoptotic signaling induced by celastrol was not blocked by newly synthesized Bcr-Abl protein.

Since Bcr-Abl degradation and apoptosis induction occurred almost simultaneously after the cells were exposed to celastrol, we then asked whether a short time exposure to celastrol is sufficient to trigger apoptosis or a sustained stimulus from celastrol is required to induce apoptosis execution. For this purpose, K-562 cells were treated with 1 μM celastrol for 1, 2, 3, 4 or 5 hours. After the treatment, half of the cells was harvested while another half was washed to remove the drug and then put back into fresh media without celastrol for another 12 hour incubation. This extra 12 hours would give cell enough time to continue the apoptotic signal transduction and execution.

As shown in Fig. 2, in each pair comparing to the cells harvested immediately after drug exposure, the cells experienced additional 12 hour incubation without celastrol, the expression of Bcr-Abl mRNA decreased.

Fig. 1. Kinetic study on Bcr-Abl degradation and apoptosis induced by celastrol in K-562 cells. K-562 cells were exposed to 1 µM celastrol for the indicated times. (A) Total cell numbers were counted manually by hemocytometer. Bars, SD. (B) Detection of caspase-3 activity in cell lysates by using fluorogenic substrate. Columns, mean of triplicates; Bars, SD. (C) Cell lysates were separated by SDS-PAGE and analysed by Western Blot using Abl and PARP antibodies. β-actin was used as a loading control. ↓ indicates Bcr-Abl fragments. (D) Detection of Bcr-Abl mRNA expression by semi-quantitative RT-PCR.

Fig. 2. Effect of short-time exposure to celastrol on Bcr-Abl degradation and apoptosis induction in K-562 cells. Cells were exposed to 1 µM celastrol for 1 to 5 hours. At each time point, one flask of cells was harvested, while the other was changed to fresh media and cultured for another 12 hours. (A) Detection of caspase-3 activity in cell lysates by using fluorogenic substrate. Columns, mean of triplicates; Bars, SD. (B) Cell lysates were separated by SDS-PAGE and analysed by Western Blot using Abl and PARP antibodies. β-Tubulin was used as a loading control.
incubation in fresh media without celastrol gave much higher levels of caspase-3 activity (Fig. 2A) and PARP cleavage (Fig. 2B). These results clearly demonstrate that a short time exposure to celastrol was sufficient to trigger apoptotic execution, if further time was given. Surprisingly, opposite to the apoptotic signaling pattern in this experiment, Bcr-Abl protein level was increased in the cells experienced further incubation after removal of celastrol (Fig. 2B). One interpretation is that new Bcr-Abl protein had been synthesized after drug removal as a cell survival mechanism, but this newly synthesized Bcr-Abl protein failed to stop or reverse the already triggered apoptotic signaling.

Fig. 3. Effect of protein synthesis inhibitor CHX on celastrol-induced Bcr-Abl degradation and apoptosis induction in K-562 cells. Cells were exposed to 1.5 μM celastrol for 2 to 5 hours. After that, cells were changed to fresh media with (+) or without (-) CHX (2 μg/mL) and cultured for another 12 hours. (A) Detection of caspase-3 activity by using fluorogenic substrate. Columns, mean of triplicates; Bars, SD. (B) Cell lysates were separated by SDS-PAGE and analyzed by Western Blot using Abl and PARP antibodies. β-Tubulin was used as a loading control.

To prove the possibility that new Bcr-Abl protein is synthesized after drug removal, we used cycloheximide (CHX) at a relatively non-cytotoxic dose (2 μg/mL) to block protein synthesis. Again, K-562 cells were exposed to 1.5 μM celastrol for 2, 3, 4 or 5 hours. After drug removal, cells were incubated in fresh media with or without CHX for another 12 hours. As predicted, the cells incubated with CHX contained lower level of Bcr-Abl protein as compared to the cells incubated without CHX (Fig. 3B). Consistently, higher levels of caspase-3 activity (Fig. 3A) and PARP cleavage (Fig. 3B) were achieved in the cells incubated with CHX. Taken together, these results indicate that K-562 cells undergo committed apoptosis after a short time exposure to celastrol and removal of celastrol may cause re-expression of Bcr-Abl protein, which however cannot rescue the cell from the fate of apoptotic death.

A variety of protease inhibitors failed to inhibit celastrol-induced Bcr-Abl degradation and apoptosis.

Based on the observation of Bcr-Abl degradation under celastrol exposure, we asked which protease is responsible for this degradation. To do this, we tested whether or not a commonly used protease inhibitor or an inhibitor mixture could block celastrol-induced Bcr-Abl degradation. A panel of protease inhibitors was tested including CA074-Me (0.5 μM, cathepsin B inhibitor), pepstatin (15 μM, aspartyl protease inhibitor), aprotinin (10 μM, serine protease inhibitor), leupeptin (400 μM, prefers to inhibit cysteine proteases but also inhibit serine proteases), and NEM (15 μM, cysteine protease inhibitor). The chosen concentration of each protease inhibitor was effective but relatively non-toxic to cells. We found that none of the single protease inhibitors was able to prevent or suppress Bcr-Abl degradation induced by celastrol (Fig. 4B). Compared to the cells treated with celastrol alone in the absence of a protease inhibitor, the similar levels of Bcr-Abl degradation, caspase-3 activation and PARP cleavage were observed in the cells co-treated with celastrol and a protease inhibitor (Fig. 4A-B). NEM-treated cells exhibited even severer Bcr-Abl degradation. This is because although the concentration of NEM we used is non-toxic to normally growing cells, depletion of thiols by NEM could possibly exaggerates cellular stress/damage induced by the drug, in this case celastrol. A mixture of the protease inhibitors only slightly suppressed caspase-3 activation and PARP cleavage (Fig. 4A-B). Nevertheless, it failed to suppress Bcr-Abl degradation and rescue the cells from cell death (Fig. 4B). Therefore, neither a single protease inhibitor nor their mixture tested...
was able to suppress celastrol-induced Bcr-Abl degradation under the selected experimental conditions.

**Fig. 4.** Effect of various protease inhibitors on celastrol-induced Bcr-Abl degradation and apoptosis induction in K-562 cells. Cells were pre-incubated with either DMSO, CA074-Me (0.5 µM), pepstatin (15 µM), aprotinin (10 µM), leupeptin (400 µM) or NEM (15 µM), or a mixture of the above five inhibitors at the indicated concentrations for 4 hours, followed by exposure to 1 µM celastrol for 12 hours. (A) Detection of caspase-3 activity by using fluorogenic substrate. Columns, mean of triplicates; Bars, SD. (B) Cell lysates were separated by SDS-PAGE and analysed by Western Blot using Abl and PARP antibodies. β-Tubulin was used as a loading control. ← indicates Bcr-Abl fragment.

**DISCUSSION**

In an attempt to effectively treat imatinib-resistant Bcr-Abl-positive leukemia, new strategies such as to decrease the amount of Bcr-Abl protein instead of to inhibit its kinase activity have been investigated. Here, we reported that 1 µM celastrol caused time-dependent reduction of Bcr-Abl protein and induction of apoptosis in K-562 cells. This result is consistent with the study from Lu et al [17]. The reduction of Bcr-Abl protein was due to protein degradation rather than transcriptional suppression (Fig. 1). Interestingly, different from celastrol, other two active compounds isolated from Celastraceae family, triptolide and pristimerin (which is celastrol methyl ether), have been reported to induce the loss of Bcr-Abl at both mRNA and protein levels [18-20]. Investigation on detailed molecular mechanisms of celastrol versus triptolide or pristimerin should be conducted in the future.

In the ensuing study on the celastrol-induced apoptotic signaling, we found that apoptotic signal triggered by celastrol within several hours was sufficient to cause apoptosis execution and cell death (Fig. 2). This finding has great clinical significance because unlike in cultured cells, in the body drugs will be metabolized and eliminated after a certain period; a quick action ensures the drug exert its effect before elimination. After removal of celastrol, the amount of Bcr-Abl protein increased slightly but steadily due to active Bcr-Abl synthesis (Fig. 2). Bcr-Abl is under the transcriptional control of Bcr promoter. In a recent study, the presence of an *"in trans"* deregulated transcription of both Bcr and Bcr-Abl promoter has been reported, which is associated with CML progression from chronic phase to blast crisis [21]. In such a scenario, it would not be surprised to see enhanced Bcr-Abl synthesis activity under intracellular stress as a cell survival mechanism. Whether the enhanced Bcr-Abl synthesis is due to increased RNA transcription and/or increased protein translation remains to be addressed. Nevertheless, this survival response as an upstream event failed to rescue K-562 cells from the fate of apoptotic cell death (Fig. 3). These results implied an effective and efficient action of celastrol.

Both caspase-mediated and cathepsin-mediated Bcr-Abl cleavage has been reported under different stimuli [22-23]. In our experimental conditions, neither a single protease inhibitor to cysteine, serine or aspartyl proteases nor a mixture of these protease inhibitors was able to prevent or suppress celastrol-induced Bcr-Abl degradation (Fig. 4). Another possibility is that Bcr-Abl is degraded by a pro-apoptotic protease such as a caspase family member. However, it has been reported that pristimerin-induced decrease of Bcr-Abl protein was not rescued by the presence of caspase inhibitor [20]. Our preliminary data showed a similar result (data not shown). It is therefore possible that the Bcr-Abl cleavage enzyme mobilized by celastrol is novel which cannot be fully inhibited by any of the protease inhibitors we tested. The cleavage sites of Bcr-Abl and the function of Bcr-Abl cleaved fragments need further investigation.
CONCLUSION

In conclusion, our studies demonstrated that celastrol induced time-dependent Bcr-Abl degradation and apoptosis in K-562 cells; even a short time exposure of cells to celastrol was sufficient to induce apoptosis execution, which was not inhibited by synthesis of new Bcr-Abl protein; celastrol-induced Bcr-Abl degradation was overwhelming which was not prevented by protease inhibitors tested. These findings contribute to the understanding of mechanisms how celastrol inhibits Bcr-Abl oncoprotein function, and support the potential application of celastrol in the CML treatment.

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